Antisense-RNA-mediated plasmid copy number control in pCG1-family plasmids, pCGR2 and pCG1, in *Corynebacterium glutamicum*

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pCGR2 and pCG1 belong to different subfamilies of the pCG1 family of *Corynebacterium glutamicum* plasmids. Nonetheless, they harbour homologous putative antisense RNA genes, *crrl* and *cgrl*, respectively. The genes in turn share identical positions complementary to the leader region of their respective *repA* (encoding plasmid replication initiator) genes. Determination of their precise transcriptional start- and end-points revealed the presence of short antisense RNA molecules (72 bp, *Crrl*; and 73 bp, *Cgrl*). These short RNAs and their target mRNAs were predicted to form highly structured molecules comprising stem-loops with known U-turn motifs. Abolishing synthesis of *Crrl* and *Cgrl* by promoter mutagenesis resulted in about sevenfold increase in plasmid copy number on top of an 11-fold (*Crrl*) and 32-fold (*Cgrl*) increase in *repA* mRNA, suggesting that *Crrl* and *Cgrl* negatively control plasmid replication. This control is accentuated by *parB*, a gene that encodes a small centromere-binding plasmid-partitioning protein, and is located upstream of *repA*. Simultaneous deactivation of *Crrl* and *parB* led to a drastic 87-fold increase in copy number of a pCGR2-derived shuttle vector. Moreover, the fact that changes in the structure of the terminal loops of *Crrl* and *Cgrl* affected plasmid copy number buttressed the important role of the loop structure in formation of the initial interaction complexes between antisense RNAs and their target mRNAs. Similar antisense RNA control systems are likely to exist not only in the two *C. glutamicum* pCG1 subfamilies but also in related plasmids across *Corynebacterium* species.

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INTRODUCTION

Plasmids are extrachromosomal, circular DNA molecules that replicate independently of chromosomal DNA. They typically possess specific DNA regions for their autonomous replication: an origin of replication (*ori*), a replication initiator protein (Rep; with a few exceptions where host-encoded proteins are used) and plasmid-harboured genes involved in replication control. As binding of Rep to *ori* is the first step of the replication process, availability of the rate-limiting Rep protein determines the replication frequency (and thus plasmid copy number) (del Solar *et al.*, 1998). Plasmids have characteristic copy numbers within given cells. To maintain the copy number, the initiation step of plasmid replication is controlled either by negative regulation mechanisms involving antisense RNAs (with or without the joint action of a transcriptional repressor) or by iterons (del Solar *et al.*, 1998). Antisense

RNAs negatively regulating replication of bacterial plasmids are typically small (50–250 bp), short-lived, constitutively synthesized, *cis*-encoded, single-stranded RNA molecules that are complementary to the 5' leader region of target mRNAs (Brantl, 2002; Khan, 1997; Rasooly & Rasooly, 1997). The currently known antisense-RNA-mediated control mechanisms include transcription attenuation (Heidrich & Brantl, 2007; Le Chatelier *et al.*, 1996; Novick *et al.*, 1989), translation inhibition (del Solar & Espinosa, 2000), inhibition of primer maturation (Davison, 1984) and inhibition of pseudoknot formation (Athanasopoulos *et al.*, 1999; Siemerling *et al.*, 1994). Antisense RNAs and their target mRNAs are generally highly structured, containing stem-loop(s). Initial interaction of antisense RNAs and their target mRNAs often involves base pairing of two complementary loop pairs (kissing complex formation) (Brantl, 2007; Franch & Gerdes, 2000). Recognition loops of antisense RNAs and their target mRNAs often possess a common structural U-turn motif [such as YUNR(UNR)- and GNRA-type consensus; Gutell *et al.*, 2000] which mediates fast RNA pairing (Franch & Gerdes, 2000; Franch *et al.*, 1999).

A number of plasmids have been isolated from the high-GC, Gram-positive industrial amino-acid-hyperproducing

Abbreviations: RT-qPCR, real-time quantitative RT-PCR; RACE, rapid amplification of cDNA ends; SD, Shine–Dalgarno.

The GenBank/EMBL/DDBJ accession number for the sequences of the *E. coli*–*C. glutamicum* shuttle vectors pCRB62 and pCRB12 are HM126493 and HM126494, respectively.

Corynebacterium glutamicum (Kinoshita *et al.*, 1957). Most of these replicate via a rolling circle mechanism and belong either to the pCG1 or the pBL1 families. A few others replicate via the theta mechanism (Tauch, 2005). The *C. glutamicum* pCG1 family includes the small cryptic plasmids pCG1 (3069 bp: Ozaki *et al.*, 1984), pGA1 (4826 bp: Sonnen *et al.*, 1991), and pSR1 (3054 bp: Archer & Sinskey, 1993) as well as larger plasmids (approx. 20–30 kb) such as pCG4, pAG1, pGA2, pTET3 and pCGR2 (Katsumata *et al.*, 1984; Sonnen *et al.*, 1991; Takeda *et al.*, 1990; Tauch *et al.*, 2002; Tsuchida *et al.*, 2010). The *C. glutamicum* pCG1 family plasmids are further divided into two distinct subfamilies based on the presence or absence of the Yux₃k/Yuxk motif of replication initiator protein (Tauch *et al.*, 2003b), which also effectively separates the subfamilies by plasmid size. The larger *C. glutamicum* pCG1 subfamily members possess a putative class Ib partitioning system (*parAB* operon) (Tauch *et al.*, 2003b) which is essential for stable plasmid segregation to daughter cells. In the whole genus *Corynebacterium*, 14 additional pCG1 family plasmids are so far known [pNG2 (Tauch *et al.*, 2003a); pBY503 (Kurusu *et al.*, 1991); pLEW279a (Williams *et al.*, 2006); pCE2 and pCE3 (Nishio *et al.*, 2003); pYM2: (GenBank accession no. AB084384); pTP10 (Tauch *et al.*, 2000); pKW4 (Tauch *et al.*, 2005); pA501 (Brune *et al.*, 2006); pK43, pK64, pCJ84 and pB85766 (Tauch *et al.*, 2004); and pA505 (GenBank accession no. NC004773). pCG1 effectively represents the largest plasmid family of this species.

Replication control in *C. glutamicum* plasmids is poorly understood except for pGA1, whose replication is negatively controlled by a small antisense RNA (Venkova-Canova *et al.*, 2003). Accessory elements of pGA1, encoded by the *per* and *aes* genes, positively influence stable maintenance of the plasmid but are not directly involved in controlling the frequency of replication initiation (Nešvera *et al.*, 1997; Venkova *et al.*, 2001). pCG1 harbours an ORF homologous to the pGA1 *per* gene (Tauch *et al.*, 2003b) but pCG1 has not been analysed in any detail.

In this study, we set out to identify and characterize antisense RNAs negatively controlling replication of two *C. glutamicum* plasmids, pCG1 and pCGR2, each of which belongs to a different pCG1 subfamily. Deletion of the partitioning gene revealed a high and robust control capacity of antisense RNA.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used are listed in Table 1. For genetic manipulation, *Escherichia coli* strains were grown at 37 °C in LB medium (Sambrook *et al.*, 1989). *C. glutamicum* was cultivated at 33 °C in 100 ml rich medium (A medium supplemented with 4% glucose; Inui *et al.*, 2007) except for the site-directed mutagenesis of *CrrI* and *CgrI*, where 10 ml rich medium was used. When appropriate, *E. coli* and *C. glutamicum* media were supplemented with 50 µg kanamycin ml⁻¹.

Molecular techniques. Restriction endonucleases were purchased from Takara. DNA ligation and kination were performed using Mighty Mix and T4 polynucleotide kinase, respectively (Takara). PCR was performed using PrimeSTAR GXL DNA Polymerase or PrimeSTAR Max DNA Polymerase (Takara) and the resulting PCR fragments were purified with the QIAquick PCR purification kit (Qiagen). *E. coli* was transformed by the CaCl₂ procedure (Sambrook *et al.*, 1989) and *C. glutamicum* by electroporation (Vertès *et al.*, 1993). DNA sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing kit with an ABI 3130xl/3730xl genetic analyser (Applied Biosystems). The sequence data were analysed using Genetyx software.

Construction of pCG1- and pCGR2-derived shuttle vectors. *E. coli*–*C. glutamicum* shuttle vectors, pCRB62 and pCRB12, derived from pCGR2 and pCG1, respectively, were constructed and deposited in GenBank with accession numbers HM126493 and HM126494, respectively (Table 1). In order to obtain a variety of pCGR2 and pCG1 derivatives (Table 1), inverse PCR was performed (i) to introduce nucleotide mutations in the –10 regions of the *cgrI* promoter (*PcgrI*) and *crrI* promoter (*PcrrI*), (ii) to delete target DNA regions (*parA*, *parB* or both), and (iii) to frameshift the *parB* gene (by exchanging 7 nt after ATG with a *SpeI* restriction site) using primers listed in Table 2. To construct pCRB62^{mt} and pCRB12^{mt}, pCRB62 and pCRB12, respectively, were used as template DNA for inverse PCR. Construction of pCRB62 derivatives with wild-type *PcrrI* used pCRB62 as template DNA, whereas derivatives with mutated *PcrrI* used pCRB62^{mt}. Inverse PCR fragments were then self-ligated and used to transform *E. coli* cells. *C. glutamicum* was transformed with the resulting constructs. *C. glutamicum* harbouring each plasmid was grown in 100 ml rich medium and 1 ml late-exponential phase (OD₆₁₀ 6.0) culture was withdrawn for extraction of total DNA and RNA.

Site-directed mutagenesis of *CrrI* and *CgrI*. Inverse PCR was performed to obtain *CrrI* and *CgrI* mutants using pCRB62(B⁻) and pCRB12 as template DNA, respectively. The *CrrI* mutants (*CrrI*-mt1 to *CrrI*-mt6) resulted from mutated forms of plasmid pCRB62(B⁻), p62(B⁻)-mt1 to p62(B⁻)-mt6, respectively (Tables 1 and 2). *CgrI* mutants, *CgrI*-mt1 to *CgrI*-mt5, resulted from mutated forms of plasmid pCRB12, p12-mt1 to p12-mt5, respectively (Tables 1 and 2). Inverse PCR fragments were then self-ligated and used to transform *E. coli* cells. *C. glutamicum* was transformed with the resulting constructs.

Nucleic acid extraction. Plasmid DNA was extracted by the alkaline lysis method (Sambrook *et al.*, 1989), or using a Miniprep kit (Qiagen) according to the manufacturer's instructions. The DNA extraction procedures were modified by pre-treating *C. glutamicum* with 4 mg lysozyme ml⁻¹ (Sigma-Aldrich) at 37 °C for 1 h.

For plasmid copy number analysis, total DNA was extracted from 1 ml late-exponential-phase (OD₆₁₀ 6.0) *C. glutamicum* cultures. The detailed procedure is described elsewhere (Tsuchida *et al.*, 2009).

For total RNA extraction, samples withdrawn from late-exponential-phase *C. glutamicum* cultures (OD₆₁₀ 6.0) were mixed with 1 vol. RNeasy Protect Bacteria Reagent (Qiagen), incubated at room temperature for 10 min, and centrifuged at 6000 g for 10 min at room temperature. The resultant pellet was resuspended in RNeasy lysis reagent (miRNeasy kit, Qiagen) and subsequently disrupted using 0.1 mm zirconia/silica beads (BioSpec Products) by eight 45 s cycles at a speed of 6.5 m s⁻¹ in a FastPrep FP100A Instrument (Q-BIOgene). After removal of the cell debris, RNA was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's instructions.

Rapid amplification of cDNA ends (RACE). The 5'- and 3'-ends of the antisense RNA genes (*crrI* and *cgrI*) and the 5'-ends of *repA* genes were localized using a SMART RACE cDNA amplification kit (Clontech). For determination of the 5'- and 3'-ends of *crrI* and *cgrI*, total RNA extracted from *C. glutamicum* harbouring pCRB12 or

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype/property	Source/reference
Strains		
<i>E. coli</i>		
HST02	F' [<i>traD36 proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^f <i>lacZ</i> ΔM15]/Δ(<i>lac-proAB</i>) <i>recA endA gyrA96 thi e14</i> [−] (<i>mcrA</i> [−]) <i>supE44 relA</i> Δ <i>deoR</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)	TAKARA
SCS110	<i>dam dcm endA1 supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr</i> Δ(<i>lac-proAB</i>)/F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^f <i>lacZ</i> ΔM15]	TOYOBO
<i>C. glutamicum</i> R	Wild-type	Yukawa <i>et al.</i> (2007)
Plasmids		
pCGR2	24 kb plasmid from <i>C. glutamicum</i>	Tsuchida <i>et al.</i> (2010)
<i>pCGR2 derivatives</i>		
pCRB62	Km ^r ; <i>E. coli</i> – <i>C. glutamicum</i> shuttle vector based on pCGR2; 5914 bp	GenBank HM126493
pCRB62(A [−])	Km ^r ; pCRB62; <i>parA</i> ORF removed	This study
pCRB62(B [−])	Km ^r ; pCRB62; <i>parB</i> ORF removed	This study
pCRB62(AB [−])	Km ^r ; pCRB62; <i>parA</i> and <i>parB</i> ORFs removed	This study
pCRB62(Bfs)	Km ^r ; pCRB62; <i>parB</i> ORF frame shifted	This study
pCRB62 ^{mt}	Km ^r ; pCRB62; <i>crrI</i> promoter mutated	This study
pCRB62 ^{mt} (A [−])	Km ^r ; pCRB62; <i>crrI</i> promoter mutated and <i>parA</i> ORF removed	This study
pCRB62 ^{mt} (B [−])	Km ^r ; pCRB62; <i>crrI</i> promoter mutated and <i>parB</i> ORF removed	This study
pCRB62 ^{mt} (AB [−])	Km ^r ; pCRB62; <i>crrI</i> promoter mutated and <i>parA</i> and <i>parB</i> ORFs removed	This study
pCRB62 ^{mt} (Bfs)	Km ^r ; pCRB62; <i>crrI</i> promoter mutated and <i>parB</i> ORF frame shifted	This study
<i>pCG1 derivatives</i>		
pCG1	3069 bp plasmid from <i>C. glutamicum</i>	Ozaki <i>et al.</i> (1984)
pCRB12	Km ^r ; <i>E. coli</i> – <i>C. glutamicum</i> shuttle vector based on pCG1; 4569 bp	GenBank HM126494
pCRB12 ^{mt}	Km ^r ; pCRB12; <i>cgrI</i> promoter mutated	This study
<i>pCRB62(B[−]) derivatives</i>		
p62(B [−])-mt1	Km ^r ; a derivative of pCRB62(B [−]) with a mutant CrrI (CrrI-mt1)	This study
p62(B [−])-mt2	Km ^r ; a derivative of pCRB62(B [−]) with a mutant CrrI (CrrI-mt2)	This study
p62(B [−])-mt3	Km ^r ; a derivative of pCRB62(B [−]) with a mutant CrrI (CrrI-mt3)	This study
p62(B [−])-mt4	Km ^r ; a derivative of pCRB62(B [−]) with a mutant CrrI (CrrI-mt4)	This study
p62(B [−])-mt5	Km ^r ; a derivative of pCRB62(B [−]) with a mutant CrrI (CrrI-mt5)	This study
p62(B [−])-mt6	Km ^r ; a derivative of pCRB62(B [−]) with a mutant CrrI (CrrI-mt6)	This study
<i>pCRB12 derivatives</i>		
p12-mt1	Km ^r ; a derivative of pCRB12 with a mutant CgrI (CgrI-mt1)	This study
p12-mt2	Km ^r ; a derivative of pCRB12 with a mutant CgrI (CgrI-mt2)	This study
p12-mt3	Km ^r ; a derivative of pCRB12 with a mutant CgrI (CgrI-mt3)	This study
p12-mt4	Km ^r ; a derivative of pCRB12 with a mutant CgrI (CgrI-mt4)	This study
p12-mt5	Km ^r ; a derivative of pCRB12 with a mutant CgrI (CgrI-mt5)	This study

pCRB62, respectively, was polyA-tailed prior to the RACE reactions, using the Poly(A)-Tailing kit (Applied Biosystems). The first strand synthesis was performed using the primers from the kit, followed by PCR (and further nested-PCR for 5'-RACE) using the gene-specific primers listed in Table 2. The gene-specific primers used for first-strand synthesis and PCRs to localize the 5'-ends of *repA* genes are listed in Table 2. The resulting PCR products were TA-cloned using the pGEM-T Easy Vector System (Promega) and sequenced.

Detection of CrrI and CgrI by RNase protection assay. Construction of antisense RNA probes and the downstream RNA hybridization/detection were performed using the *mirVana* miRNA Probe Construction kit (Ambion) and *mirVana* miRNA Detection kit (Ambion), respectively, according to the manufacturer's instructions. The DNA oligonucleotide template for transcription of antisense RNA probes was designed to cover the full *crrI* gene (72 bp) or *cgrI* gene (73 bp) plus the 8 nt sequence 5'-CCTGTCTC-3' added to the 3'-end. The DNA oligonucleotide template was then annealed to the supplied T7 promoter primer and filled in with Klenow DNA polymerase. Transcription reactions were performed using Cy-3-

labelled UTP. Total RNA (0.5 µg) extracted from late-exponential-phase cultures of *C. glutamicum* harbouring pCRB62, pCRB62^{mt}, pCRB12 and pCRB12^{mt} was hybridized with the Cy-3-labelled antisense RNA probes at 42 °C for 3.5 h, followed by RNase digestion of unhybridized RNA species and excess RNA probes. Cy-3-labelled protected RNA probe fragments were then analysed on 10% TBE-Urea gel (Novex) and visualized using a laser gel scanner (Typhoon Trio, GE Healthcare).

Real-time quantitative PCR (RT-qPCR). mRNA was quantified using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Each RT-PCR (20 µl) contained 10 µl Power SYBR Green Master Mix (Applied Biosystems), 150 nM forward/reverse primer, 5 U MuLV reverse transcriptase (Applied Biosystems), 8 U RNase inhibitor and 100 ng total RNA (1 ng total RNA for the reference 16S rRNA gene). Transcripts of the 16S rRNA and *repA* genes were quantified using primers listed in Table 2. The target gene transcripts were normalized to the reference gene transcripts (16S rRNA) from the same RNA samples. Reactions were performed as follows: 50 °C for 30 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C

Table 2. Primers used in this study

Purpose	Primer name	Sequence (5'–3')*
Inverse PCR to construct the following pCG1 and pCGR2 derivatives		
pCRB12 ^{mt}	12mt_f	AAAAATGACCCTGGCGGATT
	12mt_r	AATCCGCCAGGGTCATTTTT
pCRB62 ^{mt}	62mt_f	CAATGAGTTTGCCTTCAAAAGGACGATCAAGC
	62mt_r	ACGTGCTTTCCCCCGGGTGTCTT
pCRB62(A [−]) and pCRB62 ^{mt} (A [−])	62rmvA_f	ATGGCTGATTTATCCAAGAAGCTGG
	62rmvA_r	AAGAACACTATGACATACAAAACGTCAC
pCRB62(B [−]) and pCRB62 ^{mt} (B [−])	62rmvB_f	GTATGTTTGTATGTCATGGCATAACGAC
	62rmvB_r	CCATTAATGTTCTCCCAACAGGTTTGA
pCRB62(AB [−]) and pCRB62 ^{mt} (AB [−])	62rmvAB_f	GTATGTTTGTATGTCATGGCATAACGAC
	62rmvAB_r	AAGAACACTATGACATACAAAACGTCAC
pCRB62(Bfs) and pCRB62 ^{mt} (Bfs)	SpeI_fsB_f	TCTCACTAGTTATCCAAGAAGCTGGCGAAAA
	SpeI_fsB_r	TCTCACTAGTCATTAATGTTCTCCCAACAGGTT
RACE for the pCG1 antisense RNA gene (<i>cgrI</i>)		
5'-RACE PCR	cgr_5RACE	GTTGAACGCCCCGTGAGCGGTAACCTCAC
5'-RACE nested PCR	cgr_5RACE-N	CTCACAGGGCGTCGGCTAACCCCCAGTC
3'-RACE PCR	cgr_3RACE	CTCCCAGGTTTGGACTGGGGGTAGCCG
RACE for the pCGR2 antisense RNA gene (<i>crrI</i>)		
5'-RACE PCR	crr_5RACE	TGCATGAAGTCCGCCAGGCTTGACCCCC
5'-RACE nested PCR	crr_5RACE-N	ACCCCTGACGGACTTCGCTATCACCCGG
3'-RACE PCR	crr_3RACE	CCCGGGTGTCTCCGGGTGATAGCGA
RACE for the pCG1 <i>repA</i> gene		
First strand synthesis	12rep_1st	TGGATTCACGCCGATCCAAGCT
5'-RACE PCR	12rep_5RACE	ACGGCTGCGTGCTGCGCGTGCGTCAGA
RACE for the pCGR2 <i>repA</i> gene		
First strand synthesis	62rep_1st	CGTTTGTGCTGGCAATACCAACGATA
5'-RACE PCR	62rep_5RACE	AGCACGGCTCGTCGCGGCAAGCAGACTC
5'-RACE nested PCR	62rep_5RACE-N	GGGCGAAGAGTACCGTCCTCGTGCGCGT
qRT-PCR for quantification of the following genes		
16S rRNA gene	16S_qRT_f	CCGGTACGGCTACCTTGTTA
	16S_qRT_r	CCGTCACGTCATGAAAGTTG
<i>repA</i> (pCRB12)	12rep_qRT_f	TCAACGACAAGCTAGGAAAGC
	12rep_qRT_r	AGCCAGTCTCTCCCTCAACA
<i>repA</i> (pCRB62)	62rep_qRT_f	CTTTGGGTGAGCTGTTGGAT
	62rep_qRT_r	TACCAACGATAGGCGGTAGG
Inverse PCR mutagenesis of the CrrI region of pCRB62(B[−])		
p62(B [−])-mt1	mt1-f	TTGaACCCCTGACGGACTTCGCTA
	mt1236-r	GCCTGGCGGACTTCATGCATTTATAAGG
p62(B [−])-mt2†	mt2-f	TTcACCCCTGACGGACTTCGCTA
p62(B [−])-mt3†	mt3-f	TTcaACCCCTGACGGACTTCGCTA
p62(B [−])-mt4	mt45-f	GAGGACACCCGGGGGAAAGCA
	mt4-r	CGGGgGATAGCGAAGTCCGTCA
p62(B [−])-mt5‡	mt5-r	CGGGgGaaAGCGAAGTCCGTCA
p62(B [−])-mt6†	mt6-f	TTGCACgCCTGACGGACTTCGCTA
Inverse PCR mutagenesis of the CgrI region of pCRB12		
p12-mt1	CG-mt1-f	TCCAaACCTGGGAGAAAGCGCTCA
	CG-mt1-r	CTGGGGGTTAGCCGACGCCCTGT
p12-mt2	CG-mt23-f	CTGGGAGAAAGCGCTCAAAAATGACTCTAGC
	CG-mt2-r	GTTTcGACTGGGGGTTAGCCGACG
p12-mt3§	CG-mt3-r	GTgaGGAAGTGGGGGTTAGCCGAC
p12-mt4	CG-mt4-f	ttCCCCCAGTCCAAACCTGGGAGA
	CG-mt4-r	AGCCGACGCCCTGTGAGTTACC
p12-mt5	CG-mt5-f	CCCCAGgCCAAACCTGGGAGAAAAG
	CG-mt5-r	GTTAGCCGACGCCCTGTGAGTTAC

Table 2. cont.

*Underlining indicates the position of restriction enzyme sites; small letters indicate the position of mutagenesis.

†Primer 'mt1236-r' was used as the reverse primer.

‡Primer 'mt45-f' was used as the forward primer.

§Primer 'CG-mt23-f' was used as the forward primer.

for 30 s. The results, referred to as C_T (cycle threshold) values, were means of triplicate experiments.

Quantification of the plasmid copy number by absolute real-time quantitative PCR (absolute RT-qPCR). Plasmid copy number was calculated as the ratio of quantified *aph* (a single-copy gene on the plasmids) to *dnaA* (a single-copy gene on *C. glutamicum* R chromosome). Details of primer design to target four *dnaA* and *aph* sites, production of a standard curve and RT-qPCR analysis were as described elsewhere (Tsuchida *et al.*, 2009), except that serial dilution series of plasmids containing *dnaA* or *aph* sequence were made ranging from 1×10^4 to 1×10^8 or 1×10^5 to 1×10^9 copies μL^{-1} , respectively, to produce standard curves.

RNA secondary structure prediction. The secondary structures of the antisense RNA molecules and the target *repA* mRNAs were predicted by the RNAfold program (Hofacker *et al.*, 1994; <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>), using algorithms for calculating minimum free energy (MFE) and partition function. Isolated base pairs were avoided in the structure prediction.

RESULTS

pCG1 family plasmids pCGR2 and pCG1 harbour small antisense RNA genes in the *repA* leader region

Regions immediately upstream of the replication initiation protein genes on plasmids pCGR2 and pCG1 were investigated for the presence of antisense RNA genes. Due to the small size of antisense RNA genes, using gene-specific primers for first-strand synthesis for 5'-RACE was not feasible, meaning that total RNA had to be polyA-tailed first to extend the length of the target mRNA. The same procedure was necessary to determine the 3'-end. The result was that the <200 bp of 5'/3'-RACE products could be amplified and cloned. Thereafter nucleotide sequencing identified the transcription start- and end-points of the pCGR2 antisense RNA gene at 5 and 76 bp upstream of the *repA* initiation codon, implying the presence of a 72 bp gene (Fig. 1a). Likewise, a 73 bp gene was shown to be contained on the pCG1 plasmid with transcriptional start- and end-points at 5 and 77 bp upstream of the *repA* initiation codon (Fig. 1b). We named the gene contained on pCGR2 *crrI* and that on pCG1 *cgrI*. The transcriptional start point of the pCGR2 *repA* gene was determined to be 85 bp upstream of the initiation of the translation codon. That of the pCG1 *repA* gene was 89 bp upstream of the initiation of translation codon. Putative promoter sequences of pCGR2 *repA* (–35, 5'-GGCACA-3'; –10, 5'-TACTCT-3', Fig. 1a) and pCG1 *repA* (–35, 5'-CGC-fACA-3'; –10, 5'-TAAGCT-3', Fig. 1b) were determined

and shown to share sequence similarity with the *C. glutamicum* promoter consensus (Pátek *et al.*, 2003). The *crrI* and *cgrI* genes were therefore contained within the *repA* leader regions of their respective plasmids, but in opposite orientation to *repA*. Putative promoter regions of *crrI* (–35, 5'-GTGCTT-3'; –10, 5'-TAGGCT-3', Fig. 1a) and *cgrI* (–35, 5'-GTGTCA-3'; –10, 5'-TAGAGT-3', Fig. 1b) with sequence similarity to the *C. glutamicum* promoter consensus (Pátek *et al.*, 2003) were observed.

In order to confirm promoter activity, the –10 sequence of *crrI* on pCRB62 (pCGR2-derived shuttle vector; Table 1) was altered to 5'-CAAAC-3' to construct pCRB62^{mt} (Fig. 1a). This alteration did not change the amino acid sequence (Ser, Leu) of the complementary *repA* gene. Likewise, the *cgrI* –10 sequence on pCRB12 (pCG1-derived shuttle vector; Table 1) was altered to 5'-CAGGGT-3' to obtain pCRB12^{mt} (Fig. 1b) so that the amino acid residues (Thr, Leu) encoded by the corresponding region of complementary *repA* gene remained unchanged. Expression of antisense RNAs of pCRB62, pCRB62^{mt}, pCRB12 and pCRB12^{mt} were visualized by RNase protection assay (Fig. 2). *CrrI* and *CgrI* were detected from wild-type plasmids pCRB62 (Fig. 2a) and pCRB12 (Fig. 2b), respectively, but introducing mutation in the –10 region hindered expression of the antisense RNAs. The results clearly suggest that the putative promoter regions indeed function to express antisense RNAs.

Predicted secondary structures of *CrrI* and *CgrI* and their target mRNA leader regions

The predicted secondary structures of the antisense RNA molecules and the target *repA* mRNAs are shown in Fig. 3. *CrrI* was predicted to form a secondary structure with two stem-loops with a minimum free energy of $-41.8 \text{ kcal mol}^{-1}$ (-175 kJ mol^{-1}) (Fig. 3a). The first stem-loop consists of a GC-rich 13 bp StemI with a 5 bp terminal LoopI. The second consists of a GC-rich 13 bp StemII and an 8 bp LoopII. A sequence (5'-CUUUC-3') complementary to the putative *repA* Shine–Dalgarno (SD) sequence was located at the 5'-end of *CrrI*. LoopI (5'-UGUCC-3') and LoopII (5'-GGUGCAAG-3') of *CrrI* are complementary to a part of LoopI' (5'-GGACA-3') and LoopII' (5'-CUUGCACC-3'), respectively, of sense RNA. A GNRA U-turn motif (5'-GCAA-3') was present in LoopII of *CrrI*. LoopII' of sense RNA possessed a YUNR motif whose complementary sequence on *CrrI* LoopII (5'-CAAG-3') partially overlapped the GNRA motif (Fig. 3a).

Fig. 1. Organization of *repA* and the antisense RNA genes of pCGR2 (*crrI*) (a) and pCG1 (*cgrI*) (b). The *repA* ORFs are boxed. Asterisks indicate the antisense RNA coding regions. Transcriptional start points of *repA* are indicated by bent arrows marked +1. The putative -35, -10 and Shine-Dalgarno (SD) regions of *repA* are shaded. The putative -35 and -10 regions of antisense RNA genes are underlined. Numbers indicate the position on the native plasmid DNA. Mutations generated in this study to inactivate the antisense RNA promoters are indicated underneath the -10 sequences.

Fig. 2. Detection of CrrI (a) and CgrI (b) by RNase protection assay. (a) CrrI (72 bp) expressed from pCRB62 is shown in the left lane, and that from pCRB62^{mt} (*PcrrI* mutated) in the middle lane. Full-length probe (80 bp) is visible in the no target/no RNase control in the right lane. (b) CgrI (73 bp) expressed from pCRB12 is shown in the left lane, and that from pCRB12^{mt} (*PcgrI* mutated) in the middle lane. Full-length probe (81 bp) is visible in the no target/no RNase control in the right lane.

CgrI was also predicted to form a secondary structure with two stem-loops (Fig. 3b), with a minimum free energy of $-43.8 \text{ kcal mol}^{-1}$ (-183 kJ mol^{-1}). The first loop consists of a GC-rich 12 bp StemI with a 7 bp terminal LoopI containing the YUNR U-turn motif (5'-UUGG-3'). The second loop comprises a GC-rich 11 bp StemII with a 4 bp terminal LoopII. Similar to CrrI, the 5'-end of CgrI contained a sequence (5'-CUUUCU-3') complementary to the putative SD sequence of pCG1 *repA*. LoopI (5'-GUUUGGA-3') and LoopII (5'-UACC-3') of CgrI were complementary to LoopI' (5'-UCCAAAC-3') and a part of LoopII' (5'-GGUA-3'), respectively, of the pCG1 *repA* mRNA leader region.

CrrI and CgrI negatively control plasmid copy number

The effect of the antisense RNAs on *repA* mRNA transcription level and plasmid copy number was investigated by RT-qPCR and absolute RT-qPCR, respectively. Under the control of CrrI, the mean copy number of pCGR2-derived pCRB62 was 2.5 copies per chromosome. By inactivating *PcrrI* (pCRB62^{mt}), the copy number increased 6.6-fold, reaching a maximum of 16.6 copies per chromosome. The *repA* mRNA level of pCRB62^{mt} increased even more dramatically than plasmid copy number: it was 31.7-fold higher than that of pCRB62

Fig. 3. Predicted secondary structures of antisense RNA and the target *repA* mRNA leader regions of pCGR2 (a) and pCG1 (b), analysed by RNAfold. Putative SD sequences of *repA* are boxed with unbroken lines and marked 'SD'. The sequences complementary to the *repA* SD sequences are boxed with broken lines. Unbroken arrows indicate the start site of the *repA* genes. Broken arrows indicate regions complementary to the antisense RNA genes. Nucleotide positions are numbered on the RNA molecules. A minimum free energy (ΔG) is indicated for each secondary structure (1 kcal=4.184 kJ). The stem and terminal loop regions of the antisense RNAs CrrI and CgrI are named StemI, StemII, LoopI and LoopII. Those of the sense RNAs are named StemI', StemII', LoopI' and LoopII'. The U-turn consensus (GNRA and YUNR) motifs are indicated for the antisense and sense RNA loops.

(Fig. 4). Similar results were obtained with pCG1-derived pCRB12. Inactivation of *PcgrI* caused a 6.7-fold increase in mean plasmid copy number (from 4.6 to 30.8 copies per chromosome), and an approximately 11-fold increase in *repA* mRNA level. The results clearly show that CrrI and CgrI negatively regulate the plasmid copy number.

The partitioning gene *parB* accentuates the effect of antisense RNA on pCGR2 copy number

Negative regulation of pCGR2 replication by CrrI was clearly shown by the results described above. However, the 6.6-fold increase of pCGR2 copy number caused by inactivation of *PcrrI* was far less than the 60-fold increase

observed by Tsuchida *et al.* (2010) using a different pCGR2-derived plasmid, pCRD324. The only structural difference between these two pCGR2 derivatives is the presence in pCRB62 of a putative partitioning operon upstream of *repA* that pCRD324 lacks. Therefore, in order to investigate if the partitioning genes have any effect on the plasmid copy number, we constructed six other pCGR2 derivatives by deleting one or both of the two partitioning genes from pCRB62 and pCRB62^{mt} (Fig. 4). pCRB62(A⁻) and pCRB62^{mt}(A⁻) showed similar mRNA levels and copy numbers to pCRB62 and pCRB62^{mt}, respectively, indicating that deleting the *parA* ORF did not affect the frequency of the plasmid replication. In contrast, as observed for pCRB62^{mt}(B⁻), deleting the *parB* ORF in

Fig. 4. Construction of pCGR2 plasmid derivatives and their copy numbers and relative *repA* mRNA levels. White arrows indicate ORFs (*parA*, *parB* and *repA*). Promoters of the *parAB* operon and *repA* are indicated by unbroken bent arrows. Small grey arrows indicate the antisense RNA gene, *crrI*, whose promoters are indicated by broken bent arrows. Where *PcrrI* was inactivated by mutating the -10 region, the broken bent arrows are crossed. Black arrows indicate that the ORFs were deleted from the plasmid. Crossed arrows indicate that ParB protein was deactivated by frameshift mutation. The numbers 1592, 2119, 2418, 3193 and 4650 indicate the position on pCRB62.

the absence of CrrI resulted in a drastic increase in the plasmid copy number, which reached 218 copies per chromosome, a 73-fold increase compared with pCRB62(B⁻). Deleting both *parA* and *parB* ORFs had almost the same effect on plasmid copy number as deleting only *parB*. There was a >100-fold increase in the relative *repA* mRNA level when *parB* only, or both *parA* and *parB*, were deleted in the absence of CrrI. In order to examine if this phenomenon was caused by the functional absence of ParB, the protein was deactivated by frameshift mutagenesis. The deactivation resulted in the same effect on plasmid copy number as was observed by deleting the *parB* ORF. When the antisense RNA functioned normally, the removal of *parB* (or both *parA* and *parB*) or frameshifting *parB* resulted in relatively marginal differences in plasmid copy number and relative mRNA level (Fig. 4).

The terminal loop structure of antisense RNA plays an important role in copy number control

In order to examine the effect of antisense RNA secondary structure on plasmid replication control, site-directed mutagenesis was performed by inverse PCR. Nucleotide

substitutions were designed not to alter the SD sequence or insert extra initiation codons in the *rep* leader region. A total of six mutants, CrrI-mt1 to CrrI-mt6 (Table 3), were constructed using pCRB62(B⁻) as template, expecting the effect of site-directed mutagenesis to be most clearly observed in the absence of *parB*. Each mutant contained single or double nucleotide substitutions which were predicted to cause no major secondary structure changes of either CrrI or its target mRNA (data not shown). Minimum free energy was the same or even lower in some cases (Table 3). *C. glutamicum* was successfully transformed with plasmids containing CrrI-mt1, 2, 3 and 6. However, plasmids containing CrrI-mt4 (one internal loop disrupted) and CrrI-mt5 (both internal loops disrupted) failed to replicate. CrrI-mt1 contained one nucleotide substitution, which disrupted the GNRA motif in LoopII, but the YUNR motif in sense RNA (LoopII') remained intact. CrrI-mt2, on the other hand, comprised the intact GNRA motif (silent mutation) but with the YUNR motif in LoopII' disrupted. In CrrI-mt3 double nucleotide substitution disrupted both motifs. Plasmid copy number increased 2.5-fold upon disruption of the GNRA motif of CrrI (CrrI-mt1), whereas a lesser increase (1.6-fold) was

Table 3. Mutant forms of pCGR2 and pCG1 antisense RNAs and plasmid copy numbers

Antisense RNA	Position of nucleotide substitution	Minimum free energy (ΔG) (kcal mol ⁻¹) [kJ mol ⁻¹]	Presence of GNRA in antisense RNA loop (LoopII)	Presence of YUNR in sense RNA loop (LoopII')	Other features	Plasmid copy no.*	Plasmid copy no. increase (fold)
CrrI (WT)	–	–41.8 [–174.9]	Yes (GCAA)	Yes (CUUG)		3.0	1.0
CrrI-mt1	G50U	–41.8 [–174.9]	No (GCAA→UCAA)	Yes (CUUG)		7.6	2.5
CrrI-mt2	C51G	–41.8 [–174.9]	Yes (GCAA→GGAA)	No (CUUG→CUUC)		4.9	1.6
CrrI-mt3	G50U, C51G	–41.8 [–174.9]	No (GCAA→UGAA)	No (CUUG→CUUC)		8.1	2.7
CrrI-mt4	U27G	–48.9 [–204.6]	Yes (GCAA)	Yes (CUUG)	One internal loop closed	ND	ND
CrrI-mt5	U27G, U30A	–52.2 [–218.4]	Yes (GCAA)	Yes (CUUG)	Both internal loops closed	ND	ND
CrrI-mt6	G47C	–46.2 [–193.3]	Yes (GCAA)	YUNR→UNR motif (CUUG→UUC)	LoopII size 8 bp→6 bp	2.8	0.94
			Presence of YUNR in antisense RNA loop (LoopI)	Presence of GNRA in sense RNA loop (LoopII')			
CgrI (WT)	–	–43.8 [–183.3]	Yes (UUGG)	Yes (GUAA)		4.6	1.0
CgrI-mt1	U19G	–43.8 [–183.3]	YUNR→UNR motif (UUGG→GUGG)	Yes (GUAA)		5.0	1.1
CgrI-mt2	G21C	–43.8 [–183.3]	Yes (UUGG→UUCG)	Yes (GUAA)		5.2	1.1
CgrI-mt3	U19G, U20A	–43.8 [–183.3]	No (UUGG→GAGG)	Yes (GUAA)		6.6	1.4
CgrI-mt4	U31A, U32A	–48.3 [–202.1]	Yes (UUGG)	Yes (GUAA)	Internal loop closed	ND	ND
CgrI-mt5	A23C	–47.5 [–198.7]	Yes (UUGG)	Yes (GUAA)	LoopI size 7 bp→5 bp	11.3	2.4

*Copy number of each of four *dnaA* and *aph* targets was quantified in quadruplicate to calculate the plasmid copy number.

ND, Not determined.

observed upon disruption of the YUNR motif in target mRNA (CrrI-mt2). Removing both motifs resulted in a 2.7-fold copy number increase (CrrI-mt3), equivalent to the level observed when only the GNRA motif was disrupted. The effect of the single-strand loop size on the plasmid copy number was examined using CrrI-mt6. The size of LoopII was reduced from 8 bp to 6 bp with the GNRA motif still intact. However, shortening the loop by 2 bp did not affect plasmid copy number (0.94 relative to wild-type).

A total of five CgrI mutants were constructed (CgrI-mt1 to CgrI-mt5) by single or double nucleotide substitution (Table 3). In CgrI-mt1, 2 and 3, the nucleotide substitution was introduced within the YUNR motif in LoopI. The YUNR motif was altered to a UNR motif in CgrI-mt1, whereas a single base substitution in CgrI-mt2 was introduced in the 'N' region of YUNR, the motif remaining intact (silent mutation). Double nucleotide substitution in CgrI-mt3 completely disrupted the YUNR motif. In CgrI-mt4 the internal loop was closed by substitution of two nucleotides. In CgrI-mt5 the YUNR motif remained intact but the size of the terminal single-strand loop was reduced from 7 bp to 5 bp. Based on the RNAfold program, the basic secondary structures of the CgrI mutants and their target mRNA regions were maintained (data not shown), with the same or even lower minimum free energy (Table 3). A single exception was a major structural change in stem length of the target mRNA of CgrI-mt4: StemII' was shortened by 4 bp and StemI' became 7 bp longer, although the single-strand loops remained intact (data not shown). Again, CgrI-mt4, whose internal loop was disrupted, seemed not to be able to replicate in *C. glutamicum*, as no transformant could be obtained. Neither changing the YUNR motif to UNR nor inserting a silent mutation in YUNR resulted in any clear difference in plasmid copy number. Disrupting the YUNR motif in LoopII' of CgrI caused a moderate increase in copy number (4.6 to 6.6). In contrast to CrrI, reducing the LoopI size by 2 bp clearly hindered the inhibitory effect of CgrI, resulting in a copy number increase of about 2.4-fold (Table 3).

Antisense-RNA-mediated plasmid replication control is probably a common feature of most corynebacterial pCG1 family plasmids

Having established the presence and function of antisense RNA of pCGR2 and pCG1 we raised the hypothesis that this is a common mechanism of pCG1 family plasmids. Homology search revealed similar sequences not only in other *C. glutamicum* pCG1 family plasmids such as pTET3, pAG1 and pCG4, but also in other related plasmids from *Corynebacterium* species, including pCJ84, pA501, pK43, pK64, pKW4, pA505, pB85766, pTP10, pNG2, pYM2, pBY503 and pCE2 (Fig. 5). However, pGA2 from *C. glutamicum*, pCE3 from *Corynebacterium efficiens* and pLEW279a from *Corynebacterium* sp. L2-79-05 did not

reveal any sequences similar to those encoding antisense RNA. It is not clear whether these plasmids code for phylogenetically unrelated antisense RNA genes, or they simply do not employ antisense RNA for plasmid replication control. This observation suggests that the antisense-RNA-mediated plasmid replication control system is shared among most of the corynebacterial pCG1 family plasmids, with only a few exceptions. An unrooted dendrogram of RepA amino acid sequences from *Corynebacterium* pCG1 family plasmids with pBL1 plasmid as outgroup is shown in Fig. 6. The dendrogram comprises five distinct clusters: cluster S comprises plasmids 3–4.8 kb in size, including small pCG1-subfamily members, which do not contain partitioning genes. Clusters P1 through P4 comprise plasmids harbouring partitioning genes (the presence of partitioning genes is not known in pBY503, pTP10 and pCE3). Larger pCG1-subfamily members of *C. glutamicum* are scattered in clusters P1, P2 and P4. Cluster P3 consists solely of plasmids from *Corynebacterium* species other than *C. glutamicum*.

Plasmids classified in the same cluster based on RepA amino acid sequence similarity also showed higher sequence similarity in *repA* leader regions (Fig. 5). Antisense RNAs of all the plasmids shown in Fig. 5, except for pBY503 and pCE2, were predicted to form a secondary structure with two stem-loops. The antisense RNAs of pBY503 and pCE2 were partially homologous to other cluster P1 and P2 members, respectively, but they were longer and they were predicted to form three stem-loops. The putative –10 and –35 regions of antisense RNA genes were conserved, except that the –35 region of cluster P3 plasmids was not obvious. Even closely related antisense RNAs within the same cluster showed versatility in single-strand-loop regions (Fig. 5). In general, each antisense RNA loop contained the U-turn motif(s) and/or a full or partial sequence complementary to the U-turn motif on sense RNA; the exception was the LoopI region of cluster P1 plasmids (pCGR2, pTET3 and pNG2).

DISCUSSION

The recent isolation of a large (24 kb) *C. glutamicum* plasmid, pCGR2 (Tsuchida *et al.*, 2010), added an eighth member to the *C. glutamicum* pCG1 family plasmids pCG1, pGA1, pSR1, pTET3, pGA1, pCG4 and pGA2 (Tauch, 2005). A preliminary experiment indicated the possible involvement of antisense RNA in pCGR2 copy number control (Tsuchida *et al.*, 2010). In this study we established the existence of an antisense RNA replication control system in pCGR2 and pCG1, plasmids that belong to distinct *C. glutamicum* pCG1 subfamilies.

Plasmids pCGR2 and pCG1 expressed short antisense RNAs, CrrI and CrgI, respectively, which were somewhat shorter than that identified in pGA1 (89 bp, Venkova-Canova *et al.*, 2003). Both CgrI and CrrI were predicted to form stable secondary structures, each comprising two

Fig. 5. Comparison of the mRNA leader region of *Corynebacterium* pCG1 family plasmids. The -10 and -35 regions of the antisense RNA genes are boxed and marked '-10' and '-35', respectively. Asterisks mark identical nucleotides and dots indicate nucleotides that are semi-conserved (two different nucleotides appeared). Red, blue, orange and green letters indicate identical nucleotides among plasmids belonging to Cluster P3, P1 (excluding pLEW279a and pBY503), P2 (excluding pCE2) and S, respectively. Nucleotides of pBY503 showing homology to three other Cluster P1 members (pCGR2, pTET3 and pNG2) are coloured in pink. Nucleotides of pCE2 showing homology to two other Cluster P2 members (pAG1 and pCG4) are coloured in purple. Arrows show the position and orientation of the antisense RNA stem regions. LoopI, LoopII and LoopIII indicate the positions of terminal loops. The YUNR motif on antisense RNA is boxed. The GNRA/GNRG motif on antisense RNA is double-underlined. The complementary sequence of the YUNR motif formed on the target mRNA is boxed with broken lines. The complementary sequence of the GNRA/GNRG motif formed on the target mRNA is underlined with double broken lines. The 5'- and 3'-ends of CrrI and CgrI determined in this study, and those of pGA1 (Venkova-Canova *et al.*, 2003), are boxed and labelled either '5'' or '3'', respectively. The numbers in parentheses indicate the position on the plasmid sequence. nk, not known.

Fig. 6. Unrooted dendrogram of RepA amino acid sequences from *Corynebacterium* pCG family plasmids (pBL1 plasmid as comparison). The unrooted dendrogram comprises five distinct clusters (S and P1–P4). Plasmid names marked with (d), (e), (g), (j), (s), (L) and (st) indicate origin from *Corynebacterium diphtheriae*, *Corynebacterium efficiens*, *Corynebacterium glutamicum*, *Corynebacterium jeikeium*, *Corynebacterium striatum*, *Corynebacterium* sp. L2-79-05 and *Corynebacterium stationis* (formerly called *Brevibacterium stationis*; Bernard *et al.*, 2010), respectively. +/– indicates the presence/absence of putative partitioning genes, respectively. nk, not known. GenBank accession numbers are as follows: pCG1, BAA78091; pSR1, CAA80508; pAG1, AAD25058; pCE2, BAC19765; pCE3, BAC19786; pCG4, AAG00275; pGA1, CAA62330; pGA2, AAO18208; pTET3, CAD12209; pYM2, BAB91405; pTP10, NP_862246; pA505, NP_848176; pB85766, NP_612158; pNG2, AAM12773; pCJ84, AAL06089; pK43, AAK52805; pK64, AAL85937; pA501, AAP22001; pKW4, AAK94044; pLEW279a, YP_001096213; pBY503, E08498; pCGR2, YP_003377690; pBL1, YP_001649178.

stem-loops. The presence of a sequence complementary to the *repA* SD sequence in both CrrI and CgrI implied that the antisense RNAs act negatively at the post-transcriptional level. Given the fact that a far greater effect of CrrI and CgrI on *repA* mRNA level than on plasmid copy number was observed, it is also possible that CrrI and CgrI are involved in transcription attenuation. The mutation introduced into the pGA1 antisense RNA promoter region by Venkova-Canova *et al.* (2003) brought about a two amino acid replacement in its RepA protein, which we thought might have caused some interference in interpreting the data. However, the increase in copy number observed for pCRB62 and pCRB12 (about sevenfold) was similar to that reported for pGA1 (eightfold).

Introducing mutations into CrrI and CgrI to disrupt internal loop(s) seemed to inhibit plasmid replication in *C. glutamicum* (and thus bacterial growth in the presence of antibiotics). Given the fact that inactivating the promoter of *crrI* and *cgrI* did not affect bacterial growth, it is unlikely that this was caused by the functional absence of antisense RNA. Disruption of CrrI internal loop(s) was also predicted to eliminate the loop(s) of the target mRNA on which the *repA* SD sequence is located. As for CgrI, the *repA* SD sequence was not located on the internal loop (Fig. 3b), but disrupting the CgrI internal loop was predicted to cause a major structural change in the target

mRNA. One could therefore presume that this structural change in mRNA may, at least partially, have caused an inhibitory effect on RepA translation. Kim *et al.* (2008) reported difficulty in constructing antisense RNA mutants. As the authors suggested, this may also have been caused by some pleiotropic effects.

Binding of antisense RNA and its target mRNA often occurs through loop-loop contacts called 'kissing complexes', contacts which progress into the lower stem regions (Heidrich & Brantl, 2007). Determinants for efficient inhibition by antisense RNA include the presence of U-turn motifs as well as the size of antisense or sense RNA terminal loops (Heidrich & Brantl, 2007; Hjalt & Wagner, 1992). The antisense GNRA motif seems to play a major role as a determinant for efficient inhibition by CrrI. Disrupting the antisense YUNR motif of CgrI resulted in a lower increase in the plasmid copy number compared with CrrI. The plasmid copy number was affected differently by modifying the terminal loop size of CrrI and CgrI. Although the observed copy number increase was generally moderate in this study (maximum 2.7-fold increase), it was significant in that alteration of YUNR to UNR and an introduction of a silent mutation resulted in the same copy number as the wild-type. The results indicate that terminal loop structure characteristics, such as the U-turn motif and loop size, are important for the efficient antisense

RNA–mRNA interaction, and thus copy number control. The extent of inhibitory effect caused by each of these factors can, however, vary with each plasmid.

In the complete absence of antisense RNAs, copy numbers of the parental plasmids, pCRB62(B[−]) and pCRB12, could reach a maximum of 218 and 30.8 copies per chromosome, respectively, even though the copy number increase observed for each tested mutant was only moderate. Combinations of a variety of factors related to RNA structure are therefore probably responsible for the complete functionality of antisense RNAs.

The antisense-RNA-mediated plasmid replication control system is likely to be the prevailing feature not only in other *C. glutamicum* pCG1 family members, but also in other related plasmids from *Corynebacterium* species. Based on the phylogeny of RepA and antisense RNA it was shown that the more similar the RepA sequences, the closer the antisense RNA sequences. A lack of sequence similarity was seen only in certain regions of pCE2 antisense RNA compared with other cluster P2 members, implying that insertion of the region took place during evolution. pSR1 probably originates from pCG1 (98% sequence identity) but has partially lost the original pCG1 RepA amino acid sequence. Whether or not the *cgrI* homologue found in pSR1 actually functions in *repA* regulation is unclear. pGA2 and pCE3 were branched out in the dendrogram (cluster P4; Fig. 6), which was consistent with the absence of similar antisense-RNA-like sequences. Other than the two plasmids in cluster P4, pLEW279a was the only exception that did not contain a similar antisense-RNA-like sequence. Characteristics of the predicted single-stranded terminal loops, such as the presence of U-turn motif(s), a difference in a few nucleotides and loop size, etc., were found to be unique to each plasmid in most cases. Such differences may determine the plasmid replication control ability, which is unique to individual plasmids.

Absence of the putative gene (*parB*) encoding the centromere-binding protein caused an unexpectedly dramatic increase in *repA* mRNA levels as well as plasmid copy numbers, but only when synthesis of CrrI was abolished. The observation is unlikely to be related to the read-through transcription of the *parAB* operon into the *repA* gene, since Tsuchida *et al.* (2010) reported an increase in the copy number of a pCGR2-derived plasmid, pCRD329, from which the *parAB* promoter as well as ORFs were deleted. Since this was not caused by structural changes in the plasmid by deleting the *par* gene(s), but by functional absence of ParB protein, the following could be considered as reasons for this observation. (i) ParB proteins bind to the centromere site on plasmid DNA to form the partitioning complex, which physically limits the space for plasmid replication. The effect of the absence of ParB may become much more obvious when antisense RNA defeats its function, allowing an uncontrolled number of plasmids to co-exist within the cell. (ii) ParB serves directly

or indirectly as *rep* regulator: e.g. de la Hoz *et al.* (2000) reported the centromere-binding ω protein, which functions as a global regulator for plasmid number control in pSM19035. A study to elucidate the molecular mechanism(s) involved in this process is under way in our laboratory.

Regardless of the mechanism involved in the drastic increase in plasmid copy number upon deactivation of ParB, CrrI exerts significant negative control over plasmid replication: CrrI was able to maintain plasmid copy number at wild-type levels even in the absence of ParB. Considering that plasmid maintenance imposes a metabolic burden on the host (Ow *et al.*, 2006), such efficient control of copy number must have been crucial to sustainable evolution of plasmid DNA. The presence of putative partitioning genes seems widespread among members of pCG1 family plasmids other than the small members in cluster S, and it would be interesting to know whether such copy number increase following deletion of *parB* occurs in other plasmids as well. Manipulation of antisense RNA as well as partitioning genes may serve as a novel approach to artificially elevate the plasmid copy number for this purpose.

This study looked at phylogenetically related plasmids from the viewpoint of antisense-RNA-mediated plasmid replication control systems. Revealing the plasmid replication mechanism should not only be of scientific interest but also provide us with basic knowledge relevant to establishing better genetic engineering tools for *C. glutamicum*.

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